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Suitability of the in vitro Caco-2 assay to predict the oral absorption of aromatic amine hair dyes

Cindy Obringer [a,*], John Manwaring [a], Carsten Goebel [b], Nicola J. Hewitt [c], Helga Rothe [b]

[a] The Procter & Gamble Co., Cincinnati, OH, USA
[b] Procter & Gamble Service GmbH, Schwalbach, Germany
[c] Scientific Writing Services, Erzhausen, Germany

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ABSTRACT

Oral absorption is a key element for safety assessments of cosmetic ingredients, including hair dye molecules. Reliable in vitro methods are needed since the European Union has banned the use of animals for the testing of cosmetic ingredients. Caco-2 cells were used to measure the intestinal permeability characteristics (P_{app}) of 14 aromatic amine hair dye molecules with varying chemical structures, and the data were compared with historical in vivo oral absorption rat data. The majority of the hair dyes exhibited P_{app} values that indicated good in vivo absorption. The moderate to high oral absorption findings, i.e. ≥60%, were confirmed in in vivo rat studies. Moreover, the compound with a very low P_{app} value (APB: 3-((9,10-dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl)amino)-N,N-dimethyl-N-propyl-1-propanaminium) was poorly absorbed in vivo as well (5% of the dose). This data set suggests that the Caco-2 cell model is a reliable in vitro tool for the determination of the intestinal absorption of aromatic amines with diverse chemical structures. When used in combination with other in vitro assays for metabolism and skin penetration, the Caco-2 model can contribute to the prediction and mechanistic interpretation of the absorption, metabolism and elimination properties of cosmetic ingredients without the use of animals.

1. Introduction

The safety assessment of hair dyes naturally involves the measurement of dermal exposure since this route is the most relevant for consumer exposure: a hair color formulation containing the dye molecules is applied to the hair with access to the scalp for approximately 30 min and then washed off. However, knowledge of oral exposure is also important because the oral route is a key requirement in safety assessment in order to estimate systemic exposure with no effect levels (i.e., NOELs). The traditional in vivo model to analyze systemic exposure involved oral, intravenous (i.v.) and dermal application of a compound to the shaved skin of a rat, followed by measurement of plasma concentrations and excretion in urine and feces of the parent compound and its metabolites (OECD TG427, 2004). As part of the 7th Amendment to the Cosmetics Directive of the European Union, these in vivo rodent toxicokinetics studies were banned as of March, 2013 (EU, 2003). Therefore, reliable in vitro methods are key tools helping to provide information on absorption, metabolism and elimination needed to conduct a safety assessment. We have integrated a toolbox of in vitro models into our safety assessment strategy in order to predict skin absorption, as well as skin and hepatic metabolism properties of hair dye ingredients (Manwaring et al., 2015). Our findings showed that the combined use of in vitro absorption and metabolism assays using ex vivo skin, keratinocytes and hepatocytes have correctly predicted the toxicokinetic properties of a number of aromatic amine hair dye ingredients. Carrying on from this work, we describe here how the in vitro Caco-2 model can be applied to predict the intestinal absorption of aromatic amine hair dyes following oral exposure. When used in combination, in vitro assays predicting dermal and oral absorption as well as dermal and hepatic metabolism can provide a reliable basis to estimate the systemic exposure of the parent compound. Furthermore, the data can be particularly useful for read-across scenarios from a data-rich compound to one with limited data.

Caco-2 cells originate from a human colon carcinoma and can differentiate spontaneously into cells resembling mature small intestinal

Abbreviations: AEP, 2-amino-5-ethylphenol; CEN, 2-chloro-6-ethylamino-4-nitrophenol; HAP, 4-hydroxypropylamino-3-nitrophenol; APB, HC Blue 16; HCR, HC Red no 13; HCY, HC Yellow no 13; HDAP, 1-hydroxyethyl-4,5-diamino pyrazole; ACP, 2-amino-6-chloro-4-nitrophenol; AHT, 4-amino-2-hydroxytoluene; AMC, 4-amino-m-cresol; AMP, 6-amino-m-cresol; AME, absorption, metabolism and elimination; AUC, area under the curve; DMSO, dimethyl sulfoxide; HBSS, Hank's Balanced Salt Solution; HMA, hydroxyethyl-3,4-methylenedioxyaniline; HPD, hydroxyethyl-p-phenylenediamine; NOAEL, no adverse effect level; NOEL, no effect level; OECD, Organization for Economic Co-operation and Development; P-gp, P-glycoprotein; SCCS, Scientific Committee on Consumer Safety; TDA, toluene-2,5-diamine; TEER, transepithelial electrical resistance.

* Corresponding author.
E-mail address: obringer.cm@pg.com (C. Obringer).

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enterocytes. These cells express carrier proteins similar to the small intestine and, moreover, the TC-7 sub-clone has been shown to express the apically-located efflux pump, P-glycoprotein (P-gp) (Raeissi et al., 1999). This model is widely used to predict the absorption across the intestinal barrier (Sambuy et al., 2005; Skolnik et al., 2010), and a good correlation between oral drug absorption in humans and the apparent drug permeability (P_{app}) across the in vitro Caco-2 cell barrier has been shown (Artursson and Karlsson, 1991; Grès et al., 1998). This in vitro model was also included in the report of the 46th ECVAM workshop, identifying it as a suitable model for drug absorption (Le Ferrec et al., 2001).

The validity and suitability of such assays are typically confirmed by reference compounds for high and low absorption. In these particular studies, propranolol was used as a high permeability reference since 90% of the dose is absorbed in humans (Grès et al., 1998); and ranitidine as a low permeability reference of which only 50% of the dose is absorbed in humans. In the majority of the assays, a third reference compound, namely vinblastine, was also included as a second low absorption reference compound since its absorption is low and influenced by the presence of P-gp (Ogihara et al., 2006).

In this paper, we assess the applicability of the in vitro Caco-2 assay for cosmetics ingredients by predicting the permeability characteristics of 14 aromatic amine hair dye molecules related to phenylenediamine, aminophenol and anthraquinone based on their comparison with historical oral absorption data from rats (Fig. 1). The compounds chosen were all hair dye ingredients for which historical in vivo data were available.

2. Materials and methods

2.1. Chemicals

The hair dyes tested were TDA (toluene 2,5-diamine, CAS number 615-50-9); AMC (4-amino-m-cresol, CAS number 2835-99-6); AMP (6-amino-m-cresol, CAS number 2835-98-5); HPD (hydroxyethyl-p-phenylenediamine, CAS number 93841-25-9); HMA (hydroxyethyl-

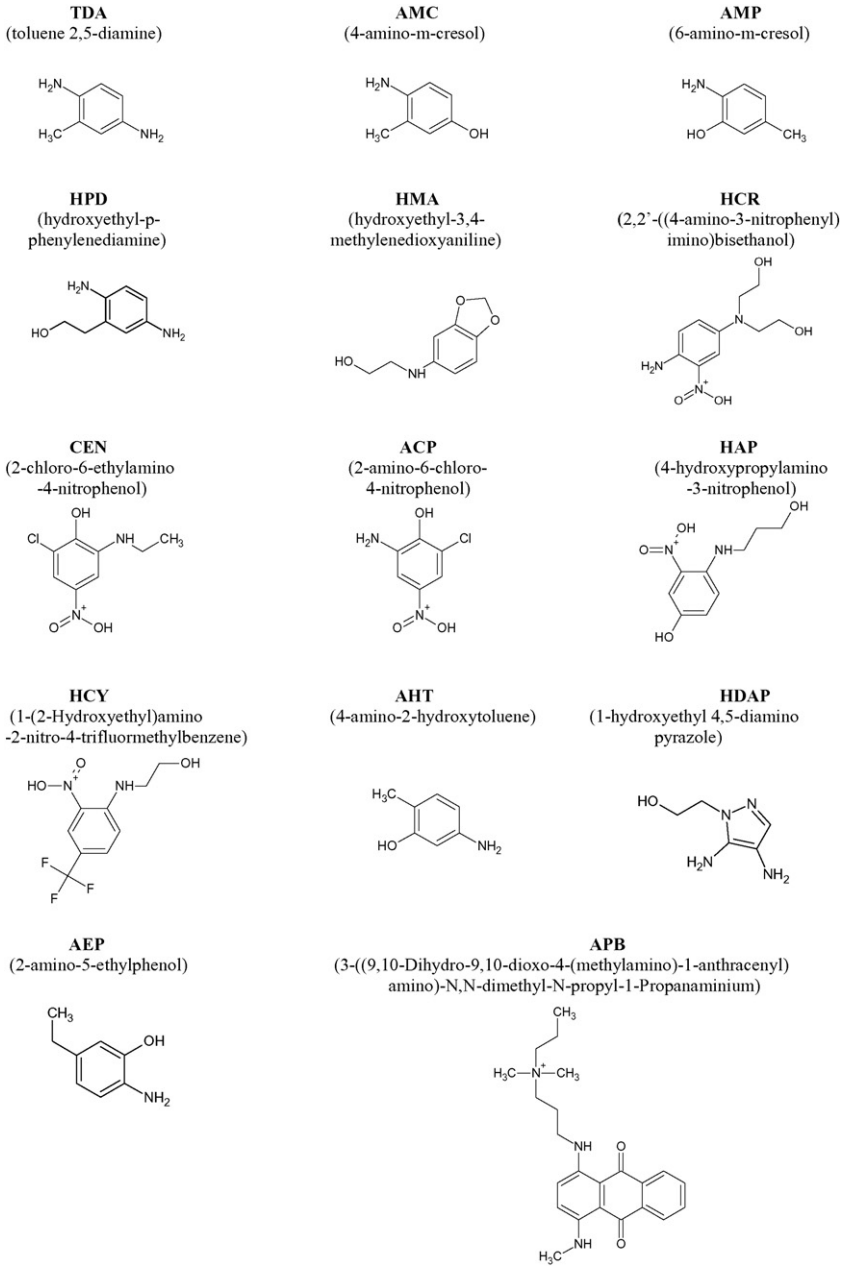

Fig. 1. Chemical structure of aromatic amine hair dye molecules similar to phenylenediamine, aminophenol and anthraquinone. All structures are shown as the free base form.

Table 1
Apical to basolateral permeability of test chemicals in Caco-2 cells. Apparent permeability values (P_{app}) were measured for reference low (vinblastine and/or ranitidine) and high (propranolol) absorption compounds, which were run at the same time as each test chemical. The values represent mean data (±SD) from two separate dosing solutions of each compound tested in one experiment, each with 3 to 6 wells independently analyzed.

Test chemical	Test chemical P_{app} ($\times 10^{-6}$ cm/s)	Low absorption control P_{app} ($\times 10^{-6}$ cm/s)		High absorption control P_{app} ($\times 10^{-6}$ cm/s)	% Absorbed test chemical P_{app}/Propranolol P^{b}_{app}
		Vinblastine	Ranitidine	Propranolol	
TDA	142.9 ± 41.5	0.06 ± 0.02	0.4 ± 0.0	47.8 ± 3.2	100 (high)
AMC	86.3 ± 38.6	0.05 ± 0.04	0.43 ± 0.04	29.6 ± 0.7	100 (high)
AMP	129.9 ± 2.2	0.05 ± 0.04	0.43 ± 0.04	29.6 ± 0.7	100 (high)
HPD	77.6 ± 20.9	0.25 ± 0.07	0.15 ± 0.07	53.1 ± 7.2	100 (high)
HMA	83.1 ± 5.7	0.49 ± 0.35	0.29 ± 0.15	34.2 ± 3.5	100 (high)
HCR	37.1 ± 2.9	0.07 ± 0.03	0.20 ± 0.00	25.9 ± 2.9	100 (high)
CEN	140.0 ± 7.5	0.06 ± 0.02	0.40 ± 0.05	47.8 ± 3.2	100 (high)
ACP	133.2 ± 0.4	0.06 ± 0.02	0.40 ± 0.05	47.8 ± 3.2	100 (high)
HAP	77.8 ± 4.1	0.07 ± 0.03	0.20 ± 0.00	25.9 ± 2.9	100 (high)
HCY	82.6 ± 5.8	0.33 ± 0.06	0.23 ± 0.04	58.3 ± 14.9	100 (high)
AHT[a]	28.2 ± 14.6	ND	1.44 ± 1.21	38.3 ± 1.7	97.5 (high)
HDAP	21.3 ± 2.1	0.49 ± 0.35	0.29 ± 0.15	34.2 ± 3.5	63.7 (moderate -high)
AEP[a]	8.9 ± 1.8	ND	1.44 ± 1.21	38.3 ± 1.7	23.3 (low–moderate)
APB[a]	1.7 ± 0.4	ND	1.44 ± 1.21	38.3 ± 1.7	3.7 (low)

Some compounds were tested in the same experiment. [a]apical pH was 7.4; [b]absorption category in parentheses; ND = not determined.

3,4-methylenedioxyaniline, CAS number 94158-14-2); HCR (2,2′-((4-amino-3-nitrophenyl)imino)bisethanol (HC red No. 13)), CAS number 94158-13-1); CEN (2-chloro-6-ethylamino-4-nitrophenol, CAS number 131657-78-8); ACP (2-amino-6-chloro-4-nitrophenol, CAS number 6358-09-4); HAP (4-hydroxypropylamino-3-nitrophenol, CAS number 92952-81-3); HCY (1-(2-hydroxyethyl)amino-2-nitro-4-trifluormethylbenzene (HC yellow No. 13), CAS number 10442-83-8); AHT (4-amino-2-hydroxytoluene, CAS number 2835-95-2); HDAP (1-hydroxyethyl 4,5-diamino pyrazole, CAS number 155601-30-2); AEP (2-amino-5-ethylphenol, CAS number 149861-22-3); and APB (3-((9,10-dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl)amino)-N,N-dimethyl-N-propyl-1-propanaminium, CAS number 502453-61-4). HPD was from MedPro Vienna, Austria. All other hair dyes were from The Procter and Gamble Service GmbH. Radiolabelled HMA was from BlyChem, Billingham, UK; radiolabelled HCR and HAP were from The Austrian Research Centre, Institute of Chemistry, Seibersdorf, Germany; and radiolabelled CEN and ACP were from Sigma Chemie, Deisenhofen, Germany. All other radiolabelled compounds were from Amersham Biosciences, Buckinghamshire, UK. All other chemicals were from Sigma-Aldrich or Applichem GmbH, Gatersleben, Germany, and were of analytical grade. Structures for the hair dyes are shown in Fig. 1. All hair dyes are referred to as the free base form, as that is the biologically relevant entity and the form present at the pH in the relevant product formulation.

2.2. Caco-2 cell culture and incubations

The Caco-2 studies were performed by external contract labs between November, 2003 and March, 2008. Caco-2 cells (sub-clones TC 7 and HTB 37) from American Type Culture Collection (Manassas, VA, USA) were seeded in 12-well or 96-well polyester Corning® Costar® transwell plates (Sigma-Aldrich, St. Louis, MO, USA) in Caco-2 medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (Life Technologies, Darmstadt, Germany), 1% non-essential amino acids (GE Healthcare Life Sciences, Pasching, Germany) and 1% gentamycin (GE Healthcare Life Sciences)). The cells were cultured for up to 25 days in a humidified incubator maintained at 37 °C in an atmosphere of 5% CO_2. The medium was renewed every 3 days. The monolayer integrity was checked every 3 days (at the time of the medium change) by measuring the transepithelial electrical resistance (TEER) until it reached a plateau of at least 280 Ω cm^2. On the day of the study, after TEER measurement, the medium was removed and

Table 2
Excretion of test chemicals in urine and feces after oral and i.v. administration. Calculation of the % oral absorption (mean ± SD) from total radioactivity in the urine (normalized to the amount recovered) and the normalized plasma AUC.

Test chemical	Dose (mg/kg)	% in urine		% in feces		Plasma dose normalized AUC (h.mg/kg/mg.kg)		% Oral absorption	
		Oral	i.v.	Oral	i.v.	Oral	i.v.	Urine	Plasma
TDA	2.5	62.2 ± 3.6	53.5 ± 6.3	31.4 ± 5.8	27.3 ± 2.1	7.10	7.46	100	95
AMC	60	92.0 ± 3.5	87.8 ± 2.5	3.9 ± 0.1	2.0 ± 0.3	2.92	3.49	98	84
AMP	25	91.3 ± 5.3	88.6 ± 2.6	4.7 ± 0.9	4.8 ± 0.4	1.92	3.46	100	55
HPD	3	82.8 ± 2.7	ND	13.6 ± 3.0	ND	ND	ND	86	ND
HMA	1	77.8 ± 8.2	78.9 ± 12.6	14.3 ± 1.4	13.1 ± 4.4	8.70	9.55	99	91
HCR	5	45.1 ± 7.5	ND	53.5 ± 7.0	ND	ND	ND	46	ND
CEN	5	62 ± 10	ND	31 ± 12	ND	ND	ND	67	ND
ACP	146	68.4 ± 4.5	ND	29.7 ± 4.6	ND	ND	ND	70	ND
HAP	0.02	67.5 ± 4.6	ND	29.3 ± 4.7	ND	ND	ND	70	ND
HCY[b]	5	82	ND	18	ND	ND	ND	82	ND
AHT	12.5	88.9 ± 1.3	93.8 ± 2.6	11.0 ± 1.2	5.7 ± 2.5	1.90	4.73	94	40
HDAP	10	73.3 ± 8.3	86.9 ± 8.0	28.2 ± 2.3	6.0 ± 2.6	2.23	3.25	77	69
AEP	370 (p.o.), 75 (i.v.)	82.9 ± 4.2	82.0 ± 3.2	10.8 ± 1.3	10.6 ± 1.5	2.41	4.73	100	51
APB	100 (p.o.), 1 (i.v.)	0.9 ± 0.5	17.5 ± 2.7	87.0 ± 2.8	67.2 ± 4.1	0.06	8.0	5	0.8–10.9[a]

[a] 0.8 is with all animals and 10.9 is with values from two animals excluded because they showed the highest concentrations compared to others in the same group; [b]data taken from SCCS dossier; ND = not determined.

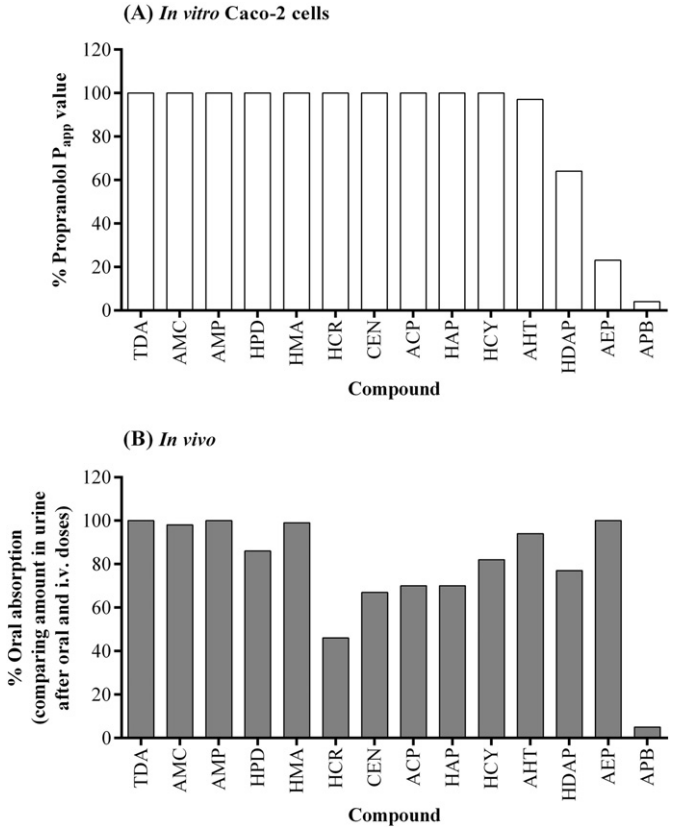

Fig. 2. A comparison of the absorption of different hair dyes through Caco-2 monolayers (A) and in vivo absorption in rats (B) using the oral and i.v. urine data. The in vitro values are expressed as a percentage of propranolol absorption (a control for high absorption). P_{app} values which were higher than the propranolol control values were set to 100% of propranolol P_{app}.

the cells were washed twice with pre-warmed (37 °C) Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) buffer to remove traces of medium.

Stock solutions were made in dimethyl sulfoxide (DMSO) or water, and further diluted in HBSS (final DMSO concentration was 1%). Each compound and reference compound (propranolol, ranitidine and/or vinblastine dissolved in HBSS) was applied to the apical side of the Caco-2 monolayer (all tested at a final concentration of 50 μM). There were two separate dosing solutions conducted for each compound, each with 3 to 6 wells independently analyzed. The pH values of the apical and basolateral compartments were 6.5 and 7.4, respectively, with the exception of AHT, AEP and APB, for which the apical pH was 7.4. After 60 min, aliquots were taken from both the apical and basolateral compartments. All samples were frozen in liquid nitrogen immediately after removal and were stored at −20 °C until analysis.

To confirm that the test chemicals did not compromise the monolayer integrity, which would cause the P_{app} value to be erroneously high, the TEER was measured again after incubation with high, medium and low absorption test chemicals (AHT, HDAP and APB, respectively). None of the three dyes affected the TEER values (data not shown). Likewise, 1% DMSO did not affect the TEER values in the monolayers (data not shown).

After thawing at room temperature, the samples were centrifuged at 1000 ×*g*, and an aliquot (90 μl) of the supernatant of each sample was transferred to a fresh analytical vial. The internal standard, chlorpropamide (10 μl 15.5 μg/ml in methanol), was then added. The samples were thoroughly mixed before analysis by LC–MS/MS.

The concentration of each compound was determined in the apical and basolateral compartments, converted to the amount in the total volume and correlated to the surface area of the transwell (to give a value in μg/cm^2). The P_{app} (cm/s) was determined using Eq. (1) (Pardridge et al., 1990):

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{m_0} \cdot \frac{1}{A} \cdot V_{Donor} \quad (1)$$

dQ: amount of substance transported (nmol or μg); dt: incubation time (3600 s); m_0: amount of substrate applied to donor compartment (nmol or μg); A: surface area of transwell membrane (cm^2); V_{Donor}: volume of the donor compartment (cm^3). To confirm that the transwell membranes had no influence on the P_{app}, negative controls without cells were run in parallel.

The values represent mean data from two separate dosing solutions of each compound tested in one experiment, each with 3 to 6 wells independently analyzed. For comparative purposes, the test compound P_{app} was divided by the corresponding propranolol P_{app} (in the same study) to give a % absorbed. Test compound P_{app} values higher than that of propranolol P_{app} values were set to a maximum of 100% (Table 1, column 6).

2.3. In vivo absorption of compounds in the rat

All historical in vivo data are from studies conducted at external contract labs between July 1988 and June 2009. All animals were handled in accordance with Dutch and EU regulations, as well as EC guidelines (EC, 1988). Animals were housed in a controlled environment at 21 ± 3 °C, 30–70% humidity and a light cycle of 12 h light and 12 h darkness per day. Rats (~200 g, Wistar CRL:WI BR) were dosed intravenously (i.v.) or orally (by gavage) with a single dose of ^{14}C-labeled test compound. Rats which were orally dosed were fasted for ~18 h prior to and ~4 h after dose administration; otherwise, all animals were allowed free access to standard pelleted laboratory animal diet (Altromin, code VRF 1, Lage, Germany). Tap-water was provided ad libitum throughout the study for all animals. In the mass-balance groups in which the animals were housed in metabolism cages, urine and feces were collected pre-dose and in 0–6, 6–12, 12–24, 24–48, 48–72 and 72–96 h intervals. In the toxicokinetic groups, blood was sampled alternately from two rats per time point at 0.25, 0.5, 1, 4, 8 and 24 h after dosing. Remaining plasma was pooled per group, and the metabolite profile in these pooled samples was investigated. Animals were euthanized 72 h or 96 h after dose administration, and several tissues and organs were collected. Total radioactivity in urine, feces, tissues and organs was determined using liquid scintillation counting. Selected urine and feces samples were pooled per group, and the metabolite profile in these pooled samples was investigated. Samples were collected, extracted and analyzed by LC–MS/MS.

Oral absorption of total radiolabelled material (i.e. parent compound and metabolites) was calculated two ways, using urine and plasma data (where available). The amount in the urine, after oral and i.v. dosing, was normalized to the total amount recovered (in the same dosing route) according to Eq. (2).

$$\text{Normalized amount in urine} = \frac{\%\text{recovered in urine}}{\%\text{recovered in urine} + \%\text{recovered in feces}} \quad (2)$$

The fraction absorbed was then calculated as the normalized amount of radioactivity recovered in the urine after oral administration divided by the normalized amount recovered after i.v. administration (Eq. (3)).

$$\text{Fraction absorbed} = \frac{\text{Normalized amount in urine(oral)}}{\text{Normalized amount in urine(i.v.)}} \quad (3)$$

This calculation assumes that the ratio of urinary excreted radioactivity to systemically available radioactivity was the same for both routes. When i.v. data were not available, the amount recovered after

i.v. administration was assumed to be 100% (Table 2, Column 6 - % Oral Absorption – Urine).

When plasma data was used, the fraction absorbed was calculated by dividing the dose normalized ^{14}C area under the curve (AUC) after oral administration by the dose normalized ^{14}C AUC after i.v. administration of the radiolabelled compound (Table 2, Column 6 - % Oral Absorption – Plasma).

3. Results and discussion

Caco-2 cells have been used for decades, most prevalently in the pharmaceutical industry, to predict the intestinal absorption of compounds (low, moderate, or high), and when used in combination with other parameters, such as solubility, this model has been shown to have a good predictive capacity (Thomas et al., 2008). P_{app} values for a particular compound can often vary significantly from lab to lab, and even somewhat from experiment to experiment. For this reason, labs will often categorize or rank compounds as having low, moderate, or high permeability based on their own historical data. We have historically used the established ranges of in vitro permeability values to predict the % absorbed (as a percentage of propranolol P_{app}): Low = 0 to ≈20%; Moderate = ≈20 to ≈60%; and High = ≈60% and higher.

We have compared the in vitro Caco-2 data for 14 hair dyes (Table 1; Fig. 2A) to the in vivo absorption properties for those same 14 hair dyes (Table 2; Fig. 2B) and found a good correlation. The P_{app} values for the 14 hair dye ingredients, together with the concurrent reference controls are shown in Table 1. Comparison of the P_{app} values of the hair dyes to the values determined for propranolol in the same study is presented in Table 1, column 6 and Fig. 2A. The reference control chemicals for low and high absorption (Table 1, columns 3, 4 and 5) confirmed that the Caco-2 cell monolayers used in these studies exhibited tight junctions (mean P_{app} values from all experiments for vinblastine and ranitidine were 0.19×10^{-6} and 0.49×10^{-6} cm/s, respectively) and also allowed the rapid permeation of the highly absorbed compound, propranolol (41.5×10^{-6} cm/s).

Of the 14 compounds, 11 compounds (TDA, AHT, AMC, AMP, HPD, HMA, HDAP, CEN, ACP, HAP, and HCY) exhibited moderate to high permeability in both the Caco-2 assay and the in vivo ADME study. Several compounds exhibited a Caco-2 permeability of 100% with a somewhat lower in vivo permeability (67–99%); however, this is still considered to be a good prediction of the permeability. Similarly, HDAP exhibited a relatively high in vivo permeability (77%) with a more moderate Caco-2 permeability (64% of propranolol P_{app}), but this is still considered predictive as both are above 60%, which typically indicates a good (moderate to high) absorption in vivo.

The remaining three compounds (APB, HCR, and AEP) exhibited a different in vitro/in vivo absorption characteristic than the high/high characteristic listed above. APB exhibited a very low in vitro Caco-2 permeability (3.7% of propranolol P_{app}) and a very low in vivo absorption (5%). HCR exhibited a high Caco-2 permeability (100% of propranolol P_{app}) with a moderate in vivo permeability (46%). AEP exhibited low Caco-2 permeability (23% of propranolol P_{app}) with a high in vivo permeability (100%).

It has been well established that physicochemical properties of a chemical can determine the extent of oral absorption. Lipinski's "rule-of-five" classifies intestinal absorption using a number of descriptors, including the LogP (Lipinski et al., 2001). APB has a LogP of 2.44 (Table 3), which being lower than the guideline of 5, indicates it should be lipophilic enough to pass into the lipid membranes, and therefore, should have good oral absorption. However, the predicted and measured intestinal absorption in Caco-2 cells and rats was much lower than the other hair dye ingredients. The P_{app} for APB was 1.47×10^{-6} cm/s, which was much lower than the corresponding P_{app} for propranolol in the same study (39.5×10^{-6} cm/s): 3.7% absorbed. The low P_{app} value for APB was reflected in the in vivo studies in which the oral absorption was only 5–11% (Table 2 and Fig. 2B). Low permeability results for some

Table 3
Test chemical, molecular weight (of the free bases), solubility and LogP values. All values were obtained from the respective SCCS opinions, with the exception of the pKa values which were calculated.

Test chemical	CAS number	Compound abbreviation	Molecular weight	Water solubility (g/l)	LogP	pKa (calculated*)	Reference
Toluene 2,5-diamine	615-50-9	TDA	122.17	5.03 (20 °C)	0.74	5.98(MB), 2.86(MB)	SCCP (2007c)
4-Amino-m-cresol	2835-99-6	AMC	123.16	12	0.51	10.34(MA), 5.13(MB)	SCCP (2007b)
6-amino-m-cresol	2835-98-5	AMP	123.16	5.9 (pH 7.65, 20 °C)	1.14	9.87(MA), 5.18(MB)	SCCS (2012b)
Hydroxyethyl-p-phenylenediamine	93841-25-9	HPD	152.18	51.2 (pH 2.02, 20 °C)	0.07	14.87(MA), 5.93(MB), 2.98(B)	SCCS (2010a)
Hydroxyethyl-3,4-methylenedioxyaniline	94158-14-2	HMA	181.25	408(pH 1.5, 20 °C) <20 (pH 6.0)	0.412 (pH 4.65, 36 °C)	14.68(MA), 5.11(MB)	SCCP (2006b)
2,2′-((4-Amino-3-nitrophenyl)imino)bisethanol (HC red No. 13)	94158-13-1	HCR	241.3	0.197 (pH 1.7, 20 °C)	0.62 (pH 7.22, RT)	14.84(A), 14.16(MA), 4.55(MB), −3.94(B)	SCCS (2010c)
2-chloro-6-ethylamino-4-nitrophenol	131657-78-8	CEN	216.62	0.105 (22 °C)	2.48 (pH 7.5, 25 °C)	6.43(MB), 2.91(B)	SCCP (2007e)
2-amino-6-chloro-4-nitrophenol	6358-09-4	ACP	188.57	0.45 (25 °C)	1.80	5.20((MA), 1.37(MB)	SCCP (2006a)
4-hydroxypropylamino-3-nitrophenol	92952-81-3	HAP	212.20	<10 (RT)	1.13 (calculated)	14.97(A), 9.43(MA), 1.08(MB)	SCCP (2007f)
1-(2-Hydroxyethyl)amino-2-nitro-4-trifluormethylbenzene (HC yellow No. 13)	10442-83-8	HCY	250.18	0.506 (pH 6.5–7.1, 23 °C)	2.54 (pH 6.5–7.1, 23 °C)	14.54(MA), −3.80(MB)	SCCS (2010b)
4-Amino-2-hydroxytoluene	2835-95-2	AHT	123.16	4.11	−0.53 (pH 7.2, RT)	10.36(MA), 4.74 (MB)	SCCP (2007d)
1-Hydroxyethyl 4,5-diamino pyrazole	155601-30-2	HDAP	142.1	666 (20 °C)	−1.75 (pH 7.0, 30 °C)	14.39(MA), 5.40(MB), −0.45(B), −5.94(B)	SCCP (2007a)
2-Amino-5-ethylphenol	149861-22-3	AEP	137.24	428 (20 °C, pH 1.42)	1.37 (pH 7.0)	9.86(MA), 5.23(MB)	SCCS (2012a)
3-((9,10-Dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl) amino)-N,N-dimethyl-N-propyl-1-Propanaminium	502453-61-4	APB	380.5	218 (pH 5.6, 20 °C)	2.44 (pH 6.0, RT)	4.23(MB), −1.50(MB)	SCCS (2012c)

RT = Room temperature, ND = not determined, * calculated using ACD Ver 12.0, A = Acid, B = Basic, MA = Mostly Acid, MB = Mostly Basic.

compounds are caused by low solubility, rather than a low permeability per se (Hoppe et al., 2014). However, we excluded this possibility because the concentrations used were well below the maximum solubility of APB in water (Table 3) and DMSO (>50 g/l (SCCS, 2012c)). The low absorption of APB may be due to the action of an efflux pump, such as P-glycoprotein (P-gp). P-gp mediates the efflux of substrates and influences their absorption, distribution and elimination, as well as functioning as a biochemical barrier for the entry of drugs across the intestine (Staud et al., 2010). In the presently reported studies, only the apical to basolateral transport was evaluated, which would not indicate if P-gp was active against the absorption of a particular compound. Evaluating the basolateral to apical transport would have provided such information; however, the effect of efflux transporters was not the main purpose of these Caco-2 studies and therefore, the B to A absorption was not included in the study design.

The P_{app} value for HCR in the Caco-2 model was higher than the propranolol P_{app}, indicating it should be well absorbed in vivo. The in vivo absorption was obtained using only oral data (no i.v. data available), which showed only 45% excreted in the urine. From this data, we know that at least 45% was absorbed, but in reality may have been higher. Excretion after an intravenous dose would have allowed a true absorption to have been determined.

Early studies testing AHT, AEP and APB were performed with the apical medium at pH of 7.4. The extent of ionization is important in determining the drug dissolution rate and passive permeability across the intestinal tract. The pH at the absorption site has been described as a potential factor affecting the dissolution and absorption of various ionizable drug molecules (DeSesso and Jacobson, 2001). Boisset found that when the pH of the apical medium was lowered from 7.4 to 6.5 in the Caco-2 assay there was an increase in permeability of the ionizable compounds tested (Boisset et al., 2000). However, the pH of the apical medium was not considered to affect the outcome of the assay since the % ionization of these dye molecules is essentially the same at pH 7.4 and 6.5 (Table 4). Subsequent experiments utilized an apical pH of 6.5 to better reflect the acid conditions of parts of the gastrointestinal tract.

The P_{app} value of AEP (8.9×10^{-6} cm/s) was significantly lower than the P_{app} value for propranolol (38.3×10^{-6} cm/s): 23% absorbed. However, the in vivo absorption of AEP was 100%. The discrepancy between the in vitro and in vivo results may be due to the low recovery of this compound in the Caco-2 assay. In the Caco-2 assay, approximately 20% of the compound was recovered while in the in vivo assay the recovery was greater than 95%. The Caco-2 assay was repeated, with a similar low recovery, showing reproducibility. Low recovery may be a result of a number of factors, such as poor solubility, non-specific binding of the compound to the plate and/or support membrane, limited metabolism by the Caco-2 cells (Pfrunder et al., 2003) or accumulation of the compound in the cell monolayer. We ruled out poor solubility since this compound was highly soluble at the concentration tested (Table 3). Additional studies would be needed to determine if AEP is accumulating in the Caco-2 cells or sticking to the plate/membrane (non-specific binding).

4. Conclusions

We have compared the in vitro and in vivo intestinal absorption properties of 14 hair dyes to confirm whether non-animal models can be used as alternatives to in vivo toxicokinetic studies as part of the safety assessment of these compounds. Overall, there was a good in vitro:in vivo correlation of the intestinal absorption such that this model identified compounds that were either well absorbed or very poorly absorbed. Of the dyes tested, the large halogenated dye, APB, was the only one that was poorly absorbed in vivo and in vitro, and this finding was in line with the known low oral absorption for similar types of molecules. Based on HDAP, for which both oral and i.v. data were available, our findings suggest that moderately to highly absorbed hair dyes may also be correctly classified by Caco-2 cells. However, more data are needed for these classes of cosmetic ingredients to determine whether they can be used to distinguish between those with moderate and extensive intestinal absorption (which has been demonstrated for drugs (Artursson and Karlsson, 1991)).

In conclusion, the data supports the use of the in vitro Caco-2 model as an alternative to in vivo studies to determine intestinal absorption of chemicals with diverse chemical structures, like hair dye molecules. When used in combination with other in vitro data using keratinocytes, hepatocytes and skin penetration data, prediction and mechanistic interpretation of the AME properties of cosmetic ingredients such as hair dyes can be made without the use of animals. Additionally, the Caco-2 assay can be used to assess historical animal data from oral dosing studies regarding applicability of the no observed adverse effect level (NOAEL) for a new compound i.e., to determine if it can be assumed that the bioavailability of the new compound is similar to that of the read-across candidate compound.

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Table 4
Effect of pH on the amount of APB, AHT, and AEP ionized.

Test chemical	% Ionized at pH 6.5	% Ionized at pH 7.4
APB 3-((9,10-Dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl) amino)-N,N-dimethyl-N-propyl-1-Propanaminium CAS 502453-61-4	99.4% (contains only one positive charge from the quaternary amine) 0.6% (contains two positive charges: one from the quaternary amines and one from the methylaniline group)	100% (contains only one positive charge from the quaternary amine)
AHT 4-Amino-2-hydroxytoluene CAS 2835-95-2	0.2% (contains one positively charged aniline group)	0%
AEP 2-Amino-5-ethylphenol CAS 149861-22-3	4.9% (contains one positively charged aniline group)	0.6% (contains one positively charged aniline group)

All ionization fractions were determined using the Henderson–Hasselbalch equation with predicted pKa values from Pipeline Pilot software version 8.5 (Biovia) (see Table 3 for pKa values).

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